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(57) Abstract

The invention relates to DNA construct comprising (i) a promoter functional in plants, (ii) a DNA sequence corresponding to an antisense polyphenol oxidase gene or part thereof, or a DNA sequence homologous thereto and (iii) a terminator functional in plants. This construct provides bruising resistance in plants.

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DNA CONSTRUCTS, CELLS AND PLANTS DERIVED THEREFROM

Scope of the invention

The invention relates to novel DNA constructs, plant cells containing these constructs and plants derived therefrom, as well as seeds obtained from said plants. In particular it involves the use of antisense nucleic acid technology to control gene expression in plants. Using tissue specific promoters the control of gene expression can be directed to particular tissues of the plant. The invention provides further, a process for obtaining plants which do not exhibit enzymatic browning of disrupted tissues.

Introduction

Browning and discolouration causes substantial losses in a wide range of fresh and processed fruits and vegetables. In potatoes, internal bruising, also called black spot, is a physiological response of potato tubers to tissue compaction and is one of the most important problems of the potato industry. Yearly, large amounts of the potato production are valueless for direct consumption or processing due to browning of the tubers.

The biochemistry of browning is relatively well understood in mammals and bacterial model systems. In plants, however very little is known about the biochemical processes involved in browning and the pathways responsible for discolouration in a wide variety of fruits and vegetables has not been elucidated. In potatoes, bruising or black spot results from minor physical damage sustained by the tubers during mechanical harvest and

transportation. The colour reaction results from a small number of cells within the bruise that actually lyse. The grey/black colour of the bruise is caused by the enzymatic production of melanin, a highly polymerised phenolic compound. The enzyme polyphenol oxidase (PPO), an oxygenase, catalyses the first two oxidation reactions of the pathway and converts monophenols (such as tyrosine) and ortho-diphenols (such as catechol) to ortho-quinones (such as dihydroxiphenylalanine-quinone). Autocatalytic chemical oxidation reactions convert ortho-quinones into melanin via several reactive quinone intermediates. Although the function of plant polyphenol oxidases has not been elucidated, several possible roles of the enzyme have been postulated. These include participation in electron transport in photosynthesis and participation in the defence mechanisms of the plant against insect attack or infections. It is believed that PPO is not involved in the synthesis of phenolic compounds in healthy, intact cells. Plant PPO enzymes are nuclear-encoded copper metalloproteins localised in membranes of plastids. In potato tubers, PPO has been localised in the rudimentary thylacoid membranes of amyloplasts. The sub-cellular location separates the enzyme from its substrate which is primarily found in the cell vacuole. However, in disrupted cells the enzyme is mixed with substrate leading to oxidation of phenolic compounds by PPO enzyme.

Although bruising has been a severe problem in the potato industry for many years, no progress has been made until now to prevent the formation of black spots into potato tubers through molecular techniques. The term bruising resistance, as used hereafter, refers to resistance against browning and discolouration upon disruption of plant cells.

We have found that bruising resistance can be obtained through interfering in the biosynthetic pathway of melanin production by the expression of an antisense PPO gene. More particularly, the production of polyphenol oxidase can be

reduced in specific tissues through tissue specific expression of said antisense PPO gene. Since the expression of antisense PPO may perturb one or more of the postulated functions of PPO, especially in aerial parts of the plant, limitation of expression to tubers, by implementation of appropriate tuber specific promoters, is preferred. Furthermore, we have found that polyphenol oxidases are encoded by a family of genes comprising at least 5 members. We have been able to suppress the expression of genes, and particularly of all genes, expressing in tubers through the coordinate expression of only one antisense PPO gene corresponding to part or entire sequence, or homologous with one of the genes encoding polyphenol oxidase. Moreover, we have been able to produce bruising resistant plants which show a reduced sensitivity to bruising, especially potato plants of which the tubers show a reduced sensitivity to bruising.

The invention is based on the application of antisense nucleic acid technology. Although the mechanism of antisense is not elucidated, it is believed that the inhibition of the expression of the target gene is obtained either through the formation of a complex of the antisense RNA and the endogenous RNA which are complementary, preventing the translation, or through the formation of a complex structure with the DNA sequence, preventing the transcription.

The invention provides a method for producing bruising resistant plants showing a reduced PPO enzyme activity and which also have been shown to have a significantly reduced browning phenotype in the field.

The invention can be used to control bruising upon disruption of tuber, vegetable, or fruit tissues in which the browning is the result of PPO enzyme activity in or around the damaged plant tissue. It is understood that the invention is not restricted to potatoes but that it can also be used for the reduction of browning upon physical tissue disruption in other

plants like leaf vegetables, i.e. lettuce, chicory or fruits, where the browning is the result of the expression of genes encoding PPO.

Detailed description of the invention

The present invention provides DNA constructs comprising i) a promoter functional in plants, ii) a DNA sequence corresponding to an antisense polyphenol oxidase gene or part thereof, or a DNA sequence homologous thereto and iii) a terminator functional in plants.

The term antisense polyphenol oxidase gene refers to a double stranded DNA sequence which sequence corresponds to or is homologous with the endogenous gene encoding PPO or part thereof, but which has a reversed orientation with respect to the said PPO gene. In a antisense DNA sequence the coding strand becomes the template strand and vice versa.

The term homologous sequence refers to a sequence in which the average nucleotide sequence homology with the contiguous gene is at least 65 %.

The term DNA sequence refers to a double stranded DNA sequence which can be expressed in plant cells if said sequence is under the expression control of a promoter functional in plants and having a terminator functional in plants.

The term promoter used herein refers to a transcription regulation region upstream from the coding sequence containing the regulatory sequences required for the transcription of the adjacent coding sequence and includes the 5' non-translated region or so called leader sequence of mRNA. The promoters used in DNA constructs according to the invention have to be functional in plant cells.

A further aspect of the invention comprises DNA constructs in which the promoter is a tissue specific promoter. A tissue specific promoter directs the transcription of the adjacent sequence in specific tissues of the plants. Suitable promoters for use in DNA constructs according to the invention include but are not limited to the cauliflower mosaic virus 35S (CaMV 35S) promoter, or potato tuber specific promoters like the promoter of the Class I Patatin gene or preferably the granule bound starch synthase gene.

The term terminator refers to a region downstream of the coding sequence which directs the termination of the transcription, also called the 3' non-translated region, which includes the poly-adenylation signal. Terminator sequence functional in plants are well known and described in the literature.

The DNA sequence according to the invention comprises at least 25 nucleotides. In general, a suitable length of the DNA sequence comprises 100 to 1000, up to 2000, nucleotides. In a preferred embodiment of the invention the DNA sequence comprises the entire coding sequence of the endogenous PPO gene, or a sequence homologous thereto, in inverted orientation.

The DNA sequence according to the invention can be derived from genomic DNA or cDNA encoding PPO. The preparation of DNA constructs according to the invention is described in more detail below. The invention also provides vectors comprising the DNA construct according to the invention.

The invention also includes plant cells comprising DNA constructs according to the invention, plants derived therefrom and seeds of such plants. Transformed plants can be obtained using standard transformation techniques like Ti-plasmid mediated transformation, direct transformation methods as particle bombardment and the like. The preferred transformation

technique to be used depends on the plant tissue and is known to the person skilled in the art.

The scope of the invention will be illustrated below, employing the construction of bruising resistant potatoes as an example. It is understood that similar approaches, according to the invention, can be used for obtaining other bruising resistant crops.

Browning of potato tubers upon physical damage can be reduced when potato plants are transformed by co-cultivation with Agrobacterium tumefaciens, comprising the DNA construct according to the invention, using internode explants and following standard protocols. In the transformed plants a reduced level of PPO activity has been measured in the tubers, whether a full length or partial sequence has been inserted in the construct in antisense orientation. The use of the tuber specific promoters preceding the DNA sequences according to the invention results in a reduction of PPO expression in the tuber but does not significantly effect the expression of PPO genes in other tissues of the plant.

In order to make DNA constructs according to the invention which will specifically target tuber expression of PPO genes, a tuber cDNA clone bank was made. Screening this bank with a probe corresponding to the sequence of a leaf PPO gene (Shahar T., Hening N., et al., The Plant Cell, 4, 135-147) or part thereof, resulted in the isolation of several different tuber specific PPO genes. At least five different genes are expressed in the potato tuber.

Further sequence analysis of the different isolated genes revealed that these genes share a nucleotide homology of at least 71,7 %. Comparison of the sequences of the tuber expressed PPO genes and PPO genes expressed in leaves showed that they are different from each other. Homology analysis with PPO genes expressed in other organisms like, bacterial, fungal

or animal species revealed that no significant homology could be found. Only conserved regions homologous to the Cu-binding sites could be identified. From these observations the conclusion can be drawn that the PPO genes of potato and in particular those expressed in the potato tuber are distinct from those identified in other species.

For obtaining bruising resistance the expression of all PPO genes expressed in the tuber should be substantially reduced. Depending on the potato cultivar used the expression should be reduced 5 to 50 fold. We have been able to obtain tubers according to the invention in which no PPO enzyme activity could be measured, using detection protocols as described hereafter, indicating that the expression of the occurring PPO genes has been inhibited in these transgenic plants. Also tubers were obtained with a reduced PPO activity with respect to the activity in the tubers of the non transformed control plants. These tubers were derived from plants transformed with antisense constructs according to the invention. Consequently the invention provides DNA constructs which causes at least a reduction of the expression of the entire PPO gene family. This multi-gene inhibition can be obtained using one antisense sequence homologous to any DNA sequence coding for PPO. We have demonstrated that at least a reduction of PPO expression can be achieved regardless of whether tuber or leaf genes were used for the construction of the antisense PPO constructs. We have also demonstrated that the antisense sequence does not have to correspond to the entire length of the coding sequence but also that partial sequences with a length of about 1200 nucleotides starting from the 5' end and including 800 nucleotides of the coding sequence, in antisense orientation, reduces the expression of the PPO genes in the tuber. Furthermore, the tubers showing a reduced PPO activity are less susceptible to bruising. In order to determine the extent of browning one can perform standardised bruising tests in which the sensitivity to bruising is expressed as a Browning Index. One test is given for instance in the following examples.

In a preferred embodiment of the invention the DNA sequence is derived from tuber PPO genes, and preferably from the PPO clones isolated from the tuber cDNA clone bank which sequences are listed in figure 1a and 1b.

In another preferred embodiment of the invention the DNA sequences in inverted orientation are preceded by a tuber specific transcription initiation region as has been set forth above. Particular criteria for the choice of a prefered tuber specific promoter which directs tuber specific expression may be summarised as follows: Expression of the antisense gene should temporally precede or at least coincide with the expression of the endogenous PPO genes. In a preferred embodiment of the invention expression of the antisense gene should be in the same tissue location as the endogenous gene. Furthermore, levels of expression should be at an appropriate level to obtain the antisense effect.

This invention can be used to control bruising upon tissue disruption of tuber, vegetable or fruit tissue in which the browning is the result of PPO enzyme activity in or around the damaged plant tissue, due to cell damage. It is understood that the invention is not restricted to potatoes but that it can also be used for the reduction browning upon bruising in other plants like leaf vegetables, i.e. lettuce, chicory or fruits, where the browning is the result of the expression of genes encoding PPO.

The invention will now be described further in the examples with reference to the accompanying drawings, in which:

Figure 1a depicts the nucleotide sequence of a PPO tuber gene. pKG45-8. This nucleotide sequence is referred to as SEQ ID NO:1.

Insertion sites :

- site A at the level of nucleotide 1
- site B at the level of nucleotide 1213

- site C at the level of nucleotide 1833.

Figure 1b depicts the nucleotide sequence of another PPO tuber gene. pKG59-4. This nucleotide sequence is referred to as SEQ ID NO: 2.

Insertion sites :

- site A at the level of nucleotide 1
- site E at the level of nucleotide 1173
- site F at the level of nucleotide 1645.

Figure 2a is a diagrammatic representation of the construction of different recombinant vectors.

Figure 2b is a diagrammatic representation of the construction of different recombinant DNA constructs used for the transformation of potato varieties.

Figure 3 is a bar diagram showing the enzyme activities of all plants transformed with antisense PPO constructs in comparison to untransformed controls. The variants denoted as 1, 2 and 3 are: transformants comprising CaMV 35S promoter constructs, transformants comprising GBSS promoter constructs, untransformed control, respectivily.

Figure 4 is a bar diagram showing the bruising indexes of selected plants transformed antisense PPO constructs in comparison to untransformed controls. The variants denoted as 1, 2 and 3 are: transformants comprising CaMV 35S promoter constructs, transformants comprising GBSS promoter constructs, untransformed control, respectivily.

The following examples illustrate the aspects of the invention, but does not restrict the scope of the invention.

Examples

Example 1: Isolation, characterisation and cloning of PPO clones from a tuber specific cDNA library

In order to make antisense constructs which will specifically inhibit expression of PPO genes in the tuber, a cDNA clone bank made from tuber RNA as starting material in the vector lambda ZAPII (Stratagene Cloning systems, 11099 North Torrey Pines Rd, La Jolla, CA 92037). The bank, comprising of 5 x 104 individual plaques, was screened with 32P labelled clone coding for a leaf PPO gene (Shahar et al. 1992). A total of 16 positively reacting plaques were taken through three rounds of plaque-purification and were chosen for further analysis.

Using PCR primers directed to the termini of the lambda ZAPII vector cloning sites, the inserts of the 16 clones were amplified and analyzed for their length and were typed according to their restriction sites. Six inserts were just over 2 kb in length, making them likely candidates for full length clones, based on work indicating that the PPO protein has a molecular weight of 45000-59000.

Example 2: Sequence analysis and detailed characterisation of potato tuber PPO cDNA clones

On the basis of the preliminary analysis of the PPO cDNA clones, two representative cDNA clones, thought to be full-length, were chosen for sequence analysis using standard sequencing protocols (pKG45-8 and pKG59-4; see Figure 1). Substrates for sequencing were produced using the in vivo excision protocol on lambda ZAPII clones (Stratagene). The remaining clones were partially sequenced in order to obtain more information about the number of individual genes being expressed in the tuber. The nucleotide sequence data revealed

two categories of cDNA clones which, although homologous, fell into two distinct classes: Class I clones (pKG45-8; Table 1) and Class II clones (pKG59-4; Table 1). The classification of the clones given in Table 1 is based on sequence homology. Interestingly the class II clones were not similar to any of the potato cDNA clones isolated and are therefore deduced to be uniquely expressed in tubers.

Amongst the 16 clones the most abundant transcript was found to belong to the same gene family (B-E) of which 10 independent cDNA clones were identified. Within this group, the B gene transcript was found most often (6 independent clones). All other transcripts (A, D and E) were present in the cDNA bank at lower levels (see Table 1).

Table 1

Genes	Class I (size in bp)	Class II (size in bp)
A	pKG45-5 (1876)	
	pKG45-8 (1850)	
B		pKG59-4 (1900)
		pKG45-6 (1850)
		pKG45-4 (1600)
		pKG45-7 (1600)
		pKG45-9 (1400)
		pKG59-1 (500)
C		pKG45-11 (1800)
		pKG45-3 (1500)
D		pKG45-10 (1300)
E		pKG59-2 (800)

Example 3: Construction of a Ti expression-vector family for constitutive and tuber specific expression.

To achieve tuber specific expression the GBSS-G28 (Rohde et

al. 1990. J. Genet. and Breed. 44: 311-315) promoter was chosen. The GBSS promoter used was isolated from genomic DNA of the potato variety Bintje (from sequence data of the genomic clone G28; Rohde, W. et al. 1990.)

Fragments containing all sequences necessary to direct tissue specificity were isolated using PCR with standard protocols. Included in the PCR primers were restriction sites to facilitate cloning into the Ti-vectors.

The GBSS promoter used (from genomic clone G28; Rohde et al. 1990), contained DNA from -1184 to -8. A HindIII site (5') and a BamHI site (3') were inserted at the termini by inclusion of the recognition sites in the PCR primers. This fragment was inserted into the gel purified Ti-vector (pKG1001; described below) after treatment of both fragment and vector with HindIII and BamHI.

The CaMV 35S expression vector was constructed from the vector pBI121 (Jefferson et al. 1987. EMBO J. 6: 3901-3907). The modifications include replacement of the mutant NPTII gene in pBI121 and the deletion of the GUS coding region; the resulting vector (pKG1001; see Figure 2a) was also the basis for the other expression vectors described below. The tuber specific promoter was inserted into pKG1001 resulting in pKG1001/GBSS containing the GBSS promoter. Figure 2a shows a diagrammatic representation of the construction of Ti-expression vectors used to make the antisense constructs.

Example 4: Cloning of antisense tuber PPO constructs using the CaMV 35S promoter and tuber specific expression Ti-vectors

Antisense constructs were made, using each of the full-length PPO genes (from pKG45-1 and pKG59-4). Another set of constructs were made using a shorter region around the translation initiation site (see Figure 1).

As a general strategy for cloning PPO genes into Ti-vectors, sequence specific PCR primers were designed against the required sites of the PPO cDNAs (see sequence, Figure 1, for precise location of sites). Incorporated into these primers were recognition sites for restriction enzymes to be used in the cloning (BamHI and BglII, 5' and 3' termini respectively).

Tuber PPO sections from pKG59-4 and pKG45-8 were inserted into the expression vectors described (pKG1001 and pKG1001/GBSS). In these experiments both the 5' segment and the full length sections from the two cDNAs were used (See sequence, Figure 1, for precise location of sites). Figure 4b shows a diagrammatic representation of the construction of the antisense PPO constructs. The names and description of the 12 resulting antisense PPO clones is shown in Table 2. The table lists the promoters used and the antisense genes inserted. Where 5' denotes the use of the first approximately 1200 nucleotides of the corresponding cDNA clone.

Table 2

Construct	Promoter	Gene
pKG60	p-CaMV 35S	- pKG45-8
pKG61	p-CaMV 35S	- pKG45-8/5'
pKG62	p-CaMV 35S	- pKG59-4
pKG63	p-CaMV 35S	- pKG59-4/5'
pKG70	p-GBSS	- pKG45-8
pKG71	p-GBSS	- pKG45-8/5'
pKG72	p-GBSS	- pKG59-4
pKG73	p-GBSS	- pKG59-4/5'

Example 5: Transformation of antisense PPO constructs into potato plants

All potato PPO constructs were introduced into Agrobacterium tumefaciens strain GV3101 via electroporation and their integrity was rechecked by restriction enzyme analysis.

Subsequently, the described constructs were introduced into two lines of potato (van Gogh and Diamant) by co-cultivation using internode explants. Standard protocols were used in these procedures. Circa 50 regenerants per construct and cultivar were grown on selective media and analyzed further.

Example 6: Analysis of PPO enzyme activities in transformed tubers

From a total of 1500 regenerants 882 were propagated and used to produce in vitro micro-tubers. From about 5 g of micro-tubers of each line a crude protein extract was produced with which PPO enzyme assays were performed (after the method of Flurkey, W. 1986 Plant Physiol. 81: 614-618). Two assays were done on each transgenic line using 50 mM catechol as a substrate for the assay in an extract volume of 1 ml. Enzyme activity is expressed as the rate of change of OD at 520 nm over 1 min at 25°C (slope). Two independent repeat measurements were performed on each line and the mean of the results were taken for analysis. On average 74% and 72% of antisense transformants in Diamant and van Gogh, respectively, gave lower PPO enzyme activity than the controls. 32 lines, harbouring the antisense PPO constructs, were found to have no detectable PPO activity with this substrate. In Diamant: 7 lines with the 35S CaMV-promoter constructs and 10 lines with the GBSS- promoter constructs showed no PPO activity. In van Gogh: 10 lines with the 35S CaMV- promoter constructs and 5 lines with the GBSS-promoter constructs had undetectable PPO activity levels.

No statistically significant differences were found between the different antisense PPO genes (Class I and II) or with respect to the size of the PPO gene-sections used in the constructs.

As shown in Figure 3, both potato varieties used showed reduced means in enzyme activity in all cases where either the 35S CaMV or GBSS promoters were used. The highest average

suppression of PPO activity was found in lines which were transformed with constructs under CaMV 35S promoter control.

Example 7: Molecular analysis of transformants

200 of the low PPO activity potato lines were further analyzed on a molecular level. Using the Southern hybridization technique it was shown that all contained T-DNA inserts of the expected sizes. Insertion copy-number ranged from 1 to 10 where 50% had copy numbers < 3. Also PPO mRNA levels were shown to be reduced when compared to untransformed controls. Assays for PPO mRNA were performed using a PCR protocol.

Example 8: Analysis of bruising resistance in the transformants

On the basis of the data from the enzyme activity, 50 lines from each variety, including all variants of antisense-PPO constructions, were selected for field trails. The selected lines represent plants with the lowest PPO enzyme activities.

In conventional breeding practice, standardised tests are carried out to determine the extent of browning in tubers from breeding lines. An index is calculated for bruising sensitivity which takes into account the level of tuber browning after subjecting the tubers to standardised mechanical damage and the specific gravity of the tubers in the test. The resulting Browning Index (BI) ranges from 0 to 50.

BI's of field grown tubers from the selected lines are shown in Figure 4. Statistical tests show significant differences in the CaMV 35S promoter series and the GBSS promoter series for both varieties when compared to the mean of the controls.

The data from these trials clearly demonstrates a significant improvement of the bruising phenotype under field

conditions as a direct result of the expression of the constructs provided by the invention.

Material and Methods

Potato plants (Solanum Tuberosum cv. van Gogh and Diamant) were grown in vitro on MS medium (Murishige, T. and Skoog, F. 1962. Physiol. Plant 15: 473 - 497) supplemented with 30g/l sucrose.

Potato internode explants were transformed with Agrobacterium tumefaciens (strain GV3101; van Larebeke, N. et al. 1974. Nature 252: 169 - 170) containing the antisense-PPO Ti-plasmid constructs using the co-cultivation method essentially according to protocols described (Ooms, G. 1989. et al. Theor. Appl. Genet 73: 744 -750).

Molecular biological methods, including Southern, Northern and Western analysis, PCR and other DNA manipulations were as described by Maniatis (Maniatis, et al. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.).

Substrates for sequencing were produced using the in vivo excision protocol on lambda ZAPII clones (Stratagene) isolated from a sink tuber cDNA.

The bruising test and the determination of the Browning Index were conducted as follows: Potatoes harvested from each line, grown in separate plots, were subjected to bruising under standard conditions. 2 - 3 kg of potatoes were placed in a shaking device comprising of a wooden box with padded walls. The box, is mechanically agitated for 2 mins. After the bruising procedure, tubers are stored for 4 days at 10°C (± 1°C). Subsequently the potatoes are mechanically peeled until 80% of the skin is removed and the degree of browning is scored

in terms of percent of the surface area effected by discolouration. The percentages are categorised into four classes and the number of tubers in each class, together with the specific gravity of the tubers is entered into the following formula from which the BI is determined:

$$BI = \frac{SG \times (CI+1) \times (CII+2) \times (CIII+3) \times (CIV+4)}{6 \times (CI+CII+CIII+CIV)} \times 100$$

Where SG is the specific gravity of the whole sample and CI - CIV are the number of tubers categorised in a given class.

Claims

1. DNA construct comprising i) a promoter functional in plants, ii) a DNA sequence corresponding to an antisense polyphenol oxidase gene or part thereof, or a DNA sequence homologous thereto and iii) a terminator functional in plants.

2. DNA construct according to claim 1 in which said promoter functional in plants is a tissue specific promoter.

3. DNA construct according to claim 2 in which said tissue specific promoter is a tuber specific promoter.

4. DNA construct according to claim 3 in which said tuber specific promoter corresponds to the promoter of the granule bound starch synthase gene.

5. DNA construct according to any of claims 1 to 4 in which said DNA sequence comprises at least 25 nucleotides.

6. DNA construct according to any of claims 1 to 5 in which said DNA sequence comprises the full length coding sequence of the polyphenol oxidase gene in antisense orientation.

7. DNA construct according to any of claims 1 to 6 in which said DNA sequence corresponds to an antisense polyphenol oxidase gene endogenous in potato plant cells or part thereof, or a DNA sequence homologous thereto.

8. DNA construct according to claim 7 in which said DNA sequence corresponds to an antisense polyphenol oxidase gene endogenous in potato tubers cells or part thereof, or a DNA sequence homologous thereto.

9. DNA construct according to any of claims 1 to 8 in which

the DNA sequence is derived from a gene coding for expression into polyphenol oxidase endogenous in plant cells.

10. DNA construct according to claim 9 in which the DNA sequence is derived from cDNA corresponding to a gene coding for expression into polyphenol oxidase endogenous in potato tuber cells.

11. DNA construct according to claim 10 in which the DNA sequence is derived from the sequence as listed in figures 1a or 1b.

12. Vector comprising a DNA construct according to any one of the preceding claims.

13. Bacterial cells comprising a vector according to claim 10.

14. Plant genome comprising a DNA construct according to any of claims 1 to 11.

15. Plant cells comprising a DNA construct according to any of claims 1 to 11.

16. Plant comprising plant cells according to claims 15.

17. Plant according to claim 16 in which the plant is a potato.

18. Seeds comprising a DNA construct according to any of claims 1 to 11.

19. Potato tubers comprising a DNA construct according to any of claims 1 to 11.

20. Process for obtaining plants having a reduced polyphenol oxidase activity, comprising the following steps: 1) inserting into the genome of plant cell comprising a DNA construct according to any of claims 1 to 11, 2) obtaining transformed

plant cells, 3) regenerating from said transformed plant cells genetically transformed plants.

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Site A         10        20        30        40        50        60
     ↓          |         |         |         |         |         |
   1 GGCAGGAGGTCTTCTTCTAGTACTACTACTCTTCCATTATGCACCAACAAATCCCTCTCT
  61 TCTTCCTTCACCACCAACAACTCATCTTTCTTATCAAAACCCTCTCAACTTTTCCTCCAC
 121 GGAAGGCGTAATCAAAGTTTCAAGGTTTCATGCAACGCCAACAATAATGTTGGCGAGCAT
 181 GACAAAAACCTTGACACTGTTGATAGGCGAAATGTTCTTTTAGGGTTAGGAGGTCTTTAT
 241 GGTGCTGCTAATCTTGCACCATTAGCCTCTGCTTCTCCTATACCACCTCCTGATCTAAAA
 301 TCTTGTGGTGTTGCCCATGTAACAGAAGGTGTTGATGTGACATATAGTTGTTGCCCTCCA
 361 GTACCCGATGATATCGATAGCGTTCCGTACTACAAGTTCCCTCCTATGACTAAACTCCGC
 421 ATCCGCCCCCCTGCTCATGCGGCGGATGAGGAGTATGTAGCCAAGTATCAATTGGCTACG
 481 AGTCGAATGAGGGAACTTGATAAAGACTCTTTTGACCCTCTTGGGTTTAAACAACAAGCT
 541 AATATTCATTGTGCTTATTGTAACGGTGCTTATAAAGTTGGTGGTAAAGAGTTGCAAGTT
 601 CATTTCTCGTGGCTTTTCTTTCCGTTTCATAGATGGTACTTGTATTTCTATGAAAGAATA
 661 TTGGGATCACTTATTAATGATCCAACTTTTGCTTTACCATATTGGAATTGGGATCATCCA
 721 AAAGGTATGCGTATACCTCCCATGTTTGATCGTGAGGGGTCATCTCTTTACGATGATAAA
 781 CGTAACCAAAACCATCGCAATGGAACTATTATTGATCTTGGTCATTTTGGTCAGGAAGTT
 841 GACACACCTCAGCTTCAGATAATGACTAATAATTTAACACTAATGTACCGTCAAATGGTC
 901 ACTAATGCTCCTTGTCCGTCCCAATTCTTCGGTGCTGCTTACCTCTGGGGACTGAACCAA
 961 GTCCAGGAATGGGTACTATTGAGAACATCCCTCATACCCCGTGCCATATCTGGACTGGTG
1021 ATAGTCCTAGACAAAAAAACGGTGAAAACATGGGTAATTTCTATTCAGCACGGTTTAGAC
1081 CCGCTTTTTTACTGTCACCACGCAAATGTGGACCGGATGTGGGATGAATGGAAATTAATT
1141 GGCGGGAAAAGAAGGGATCTATCAAATAAAGATTGGTTGAACTCAGAATTCTTTTTCTAC
                    ↓ Site B
1201 GATGAAAATCGCAACCCTTACCGTGTGAAAGTCCGTGACTGTTTGGACAGTAAAAAAATG
1261 GGATTCAGTTACGCTCCAATGCCAACTCCATGGCGTAATTTTAAACCAATCAGAAAAACT
1321 ACAGCAGGAAAAGTGAATACAGCGTCAATTGCACCAGTCACCAAGGTGTTCCCACTAGCG
1381 AACTGGGACCGTGCAATTTCGTTCTCTATCACCAGACCAGCTTCGTCAAGGACTACACAG
1441 GAGAAAAATGAGCAAGAGGAGATACTGACATTCAACAAAGTAGCCTATGATGATACTAAG
1501 TATGTAAGGTTCGATGTGTTCCTGAACGTTGACAAGACTGTGAATGCGGATGAGCTTGAT
1561 AAGGCGGAGTTTGCGGGGAGTTATACTAGCTTGCCGCATGTTCATGGAAATAATACTAAT
1621 CATGTTACGAGTGTTACTTTCAAGCTGGCGATAACAGAACTGTTGGAGGATAATGGATTG
1681 GAAGATGAAGATACTATTGCGGTAACTTTGGTTCCAAAAGTTGGTGGTGAAGGTGTATCC
1741 ATTGAAAGTGTGGAGATCAAGCTTGAGGATTGTTAAGTCCTCATGAGTTGGTGGCTATGG
                                         Site C↓
1801 TCAAATTTATGTTAATTAGTATTAAGTGTGTATTTGATTATGTTTCGGTTAAAATGTA
```

FIGURE 1a

```
Site A       10        20        30        40        50        60
   ↓          |         |         |         |         |         |
   1 CTTTTACTTCTTCCTCCACTTCTTTAACTTCCACTCCTAAACCCTCTCAACTTTTCATCC
  61 ATGGAAAACGTAACCAAATGTTCAAAGTTTCATGCATGGTTACCAATAATAACGGTGACC
 121 AAAACCAAAACGTTGAAACGAATTCTGTTGATCGAAGAAATGTTCTTCTTGGCTTAGGTG
 181 GTCTTTATGGTGTTGCTAATGCTATACCATTAGCTGCATCCGCTACTCCATCTCCACCTC
 241 CTGATCTCTCGTCTTGTAGTATAGCCAGGATTAACGAAACTCATGTGGTGCCGTACAGTT
 301 GTTGCGCGCCTAAGCCTGATGATATGGAGAAAGTTCCGTATTACAAGTTCCCTTCTATGA
 361 CTAAGCTCCGTGTTCGTCAGCCTGCTCATGAAGCTAATGAGGAGTATATTGCCAAGTACA
 421 ATTTGGCGGTTAGCAAGATGAGGGATCTTGATAAGACACAACCTTTAAACCCTATTGGTT
 481 TTAAGCAACAAGCTAATATACATTGTGCTTATTGTAACGGTGCTTATAGAATTGGTGGCA
 541 AAGAGTTACAAGTTCATAATTCATGGCTTTTCTTCCCGTTCCATAGATGGTACTTGTACT
 601 TCTACGAGAGAATCGTGGAAAACTCTTAGTAGTAGCAACTTTCGCTTTGCCTATTAGGT
 661 TAAGGGACCATCCAAAAGGTATGCGTTTTCCTGCCATGTATGATCGTGAAGGGACTTCCC
 721 TTTTCGATGTAACACGTGACCAAAGTCACCGAAATGGAGCAGTAATCGATCTTGGTTTTA
 781 TCGGCAATGAAGTCGAAACAACTCAACTCCAGTTGATGAGCAATAATTTAACACTAATGT
 841 ACCGTCAAATGGTAACTAATGCTCCATGTCCTCGGATGTTCTTTGGCGGGCCTTATGATT
 901 TCTCGGGGTTAACACTAAACTCACCGGGAACTATAGAAAACATCCCTCACGGTCCTGTCC
 961 ACTACTGGTCTGGTACAGTGACAGGTTCAAAATTTCCTAACGGTGATGTGTCCTACGGTG
1021 AGGATATGGGTAATTTCTACTCAGCTGGTTTGGACCCGGTTTTCTTTTGCCATCACAGTA
1081 ATGTGGACCGGATGTGGAGCGAATGGAAAGCGACAGGAGGGAAAAGAATAGATATCACAC
                                  Site E ↓
1141 ATAAAGATTGGTTGAACTCCGAGTTCTTTTTCTACGATGAAAATGAAAACCCTTACCGTG
1201 TGAAAGTTCGAGACTGTTTGGACACGAAGAAGATGGGTTATGATTACAAACCAATGGCCA
1261 CACCATGGCGTAACTTCAAGCCCTTAACAAAGGCTTCAGCTGGGAAAGTGAATACAACTT
1321 CACTTCCGCCAGCTAGCAATGTATTCCCATTGGCGAAGATGGATAAGACTATTTCATTTG
1381 CTATCAACAGGCCAGCTTCATCGCGGACTCAACAAGAGAAAAATGAACAAGAGGAGATGT
1441 TAACGTTCAATAACATAAGATATGATAACAGAGGGTACATAAGGTTCGATGTGTTCCTGA
1501 ACGTGGACAATGGTGTGAATGTGAATGCGAATGAGCTTGACAAGGTGGAGTTTGCGGGGA
1561 GTTATACTAGTTTGCCACATGTTCATACAGCTGGTGAGACTAATAATATCGGGACTGCTG
                                 ↓ Site F
1621 ATTTAGAGCTGGCGATAACGGAACTGTTGGAGGATATTGGTTTGGAAGAT
```

FIGURE 1b

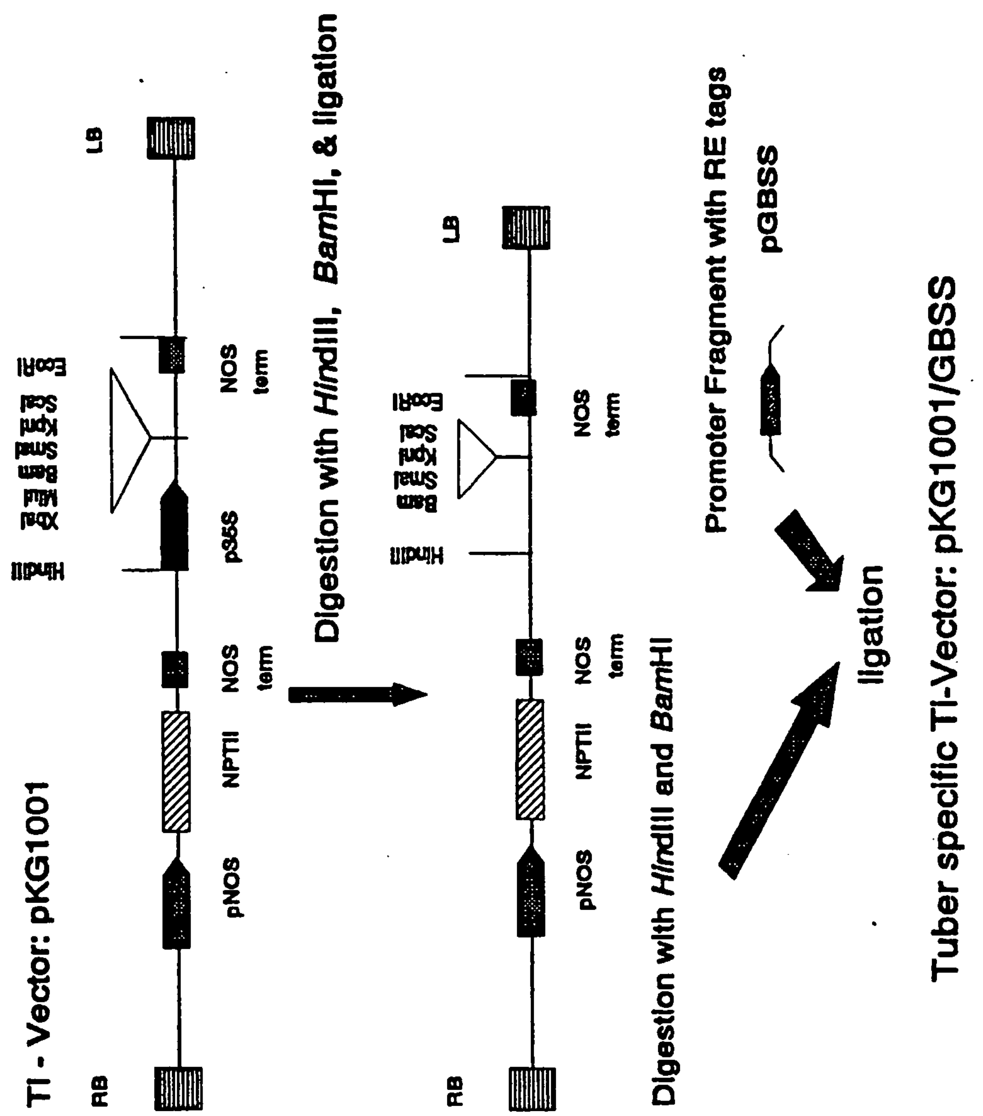
Ti - Vector: pKG1001
RB
LB
pNOS
NPTII
NOS term
p35S
NOS term
HindIII
XbaI
MluI
Bam
SmaI
KpnI
ScaI
EcoRI
Digestion with HindIII, BamHI, & ligation
RB
LB
pNOS
NPTII
NOS term
HindIII
Bam
SmaI
KpnI
ScaI
EcoRI
NOS term
Digestion with HindIII and BamHI
Promoter Fragment with RE tags
pGBSS
ligation
Tuber specific Ti-Vector: pKG1001/GBSS

FIGURE 2a

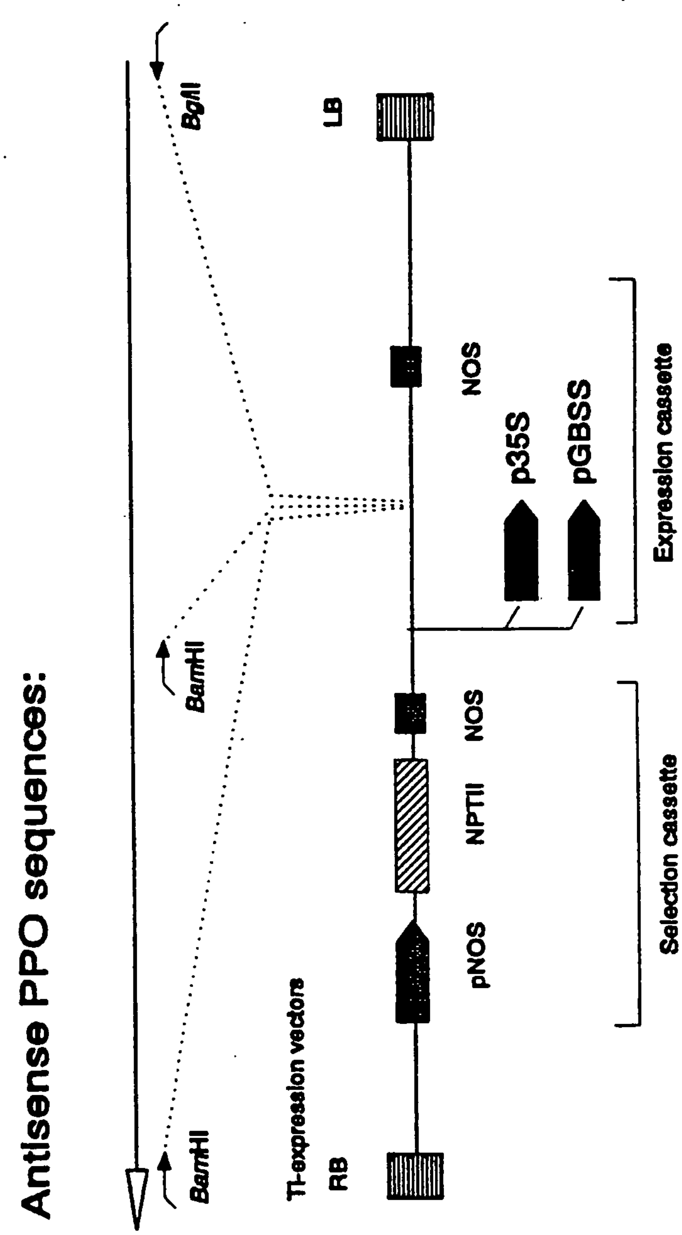
Antisense PPO sequences:
BamHI
BamHI
BglII
Ti-expression vectors
RB
pNOS
NPTII
NOS
NOS
LB
p35S
pGBSS
Selection cassette
Expression cassette

FIGURE 2b

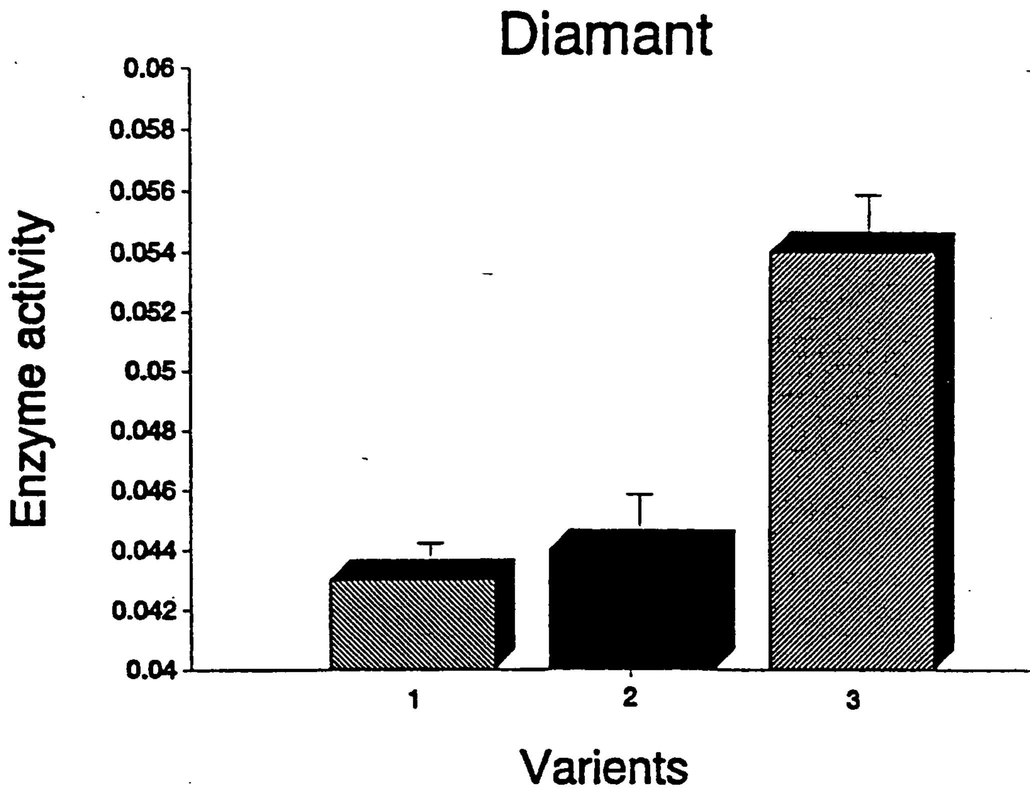

FIGURE 3A

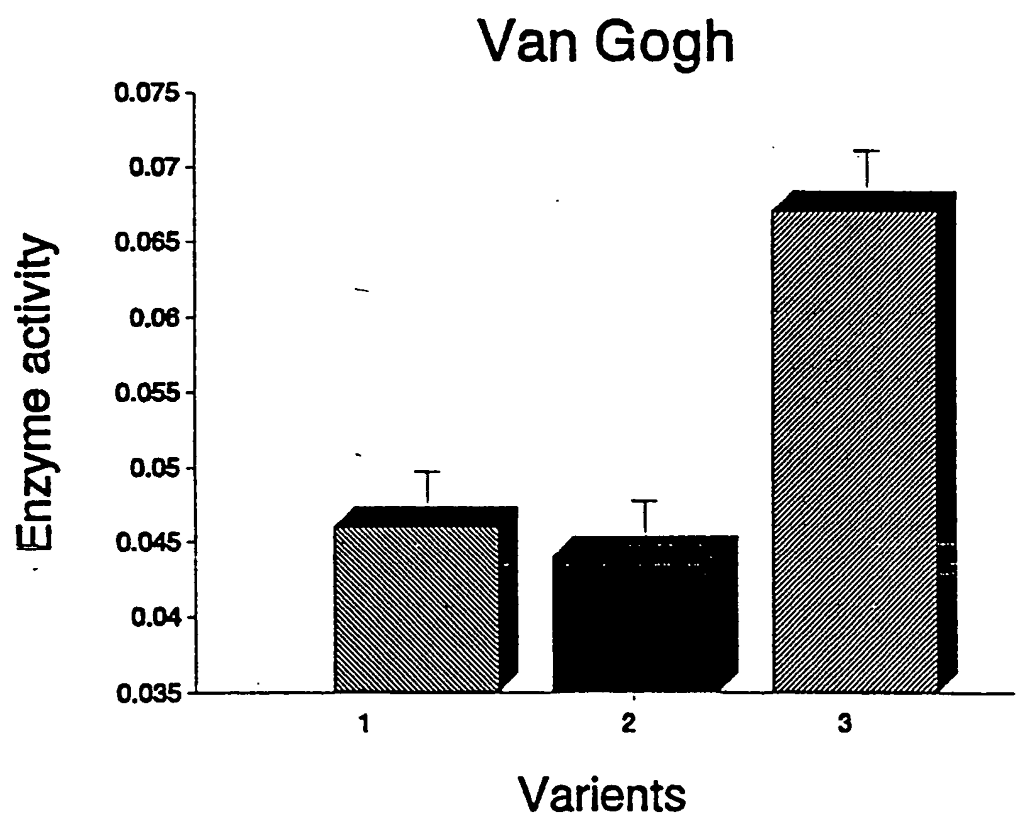

FIGURE 3B

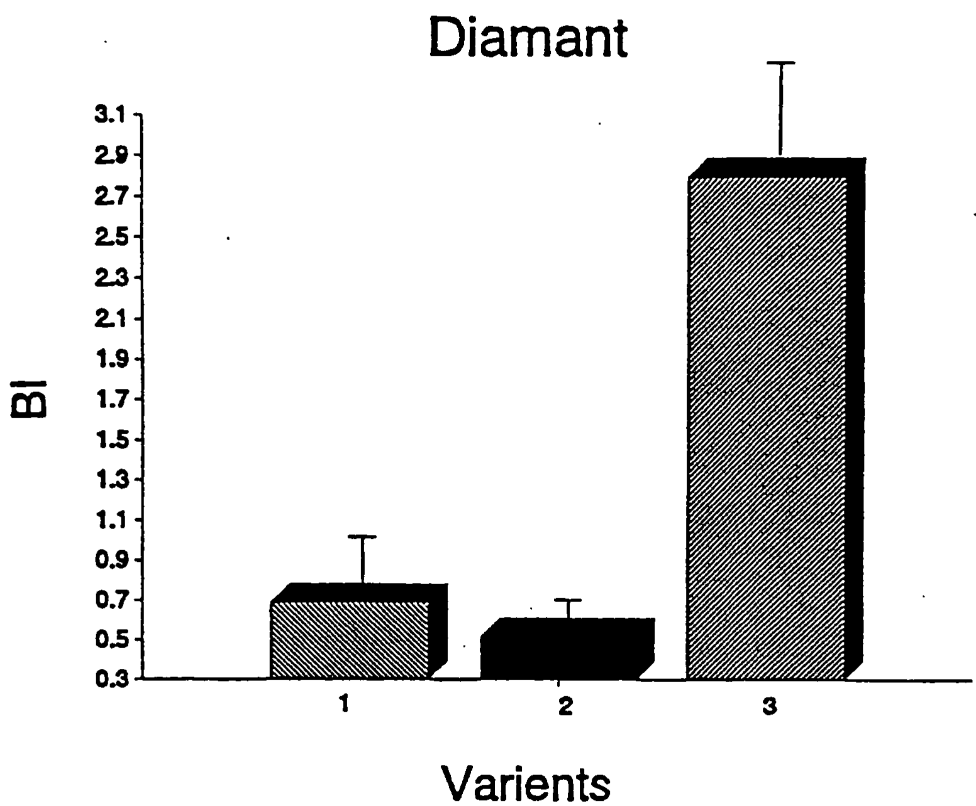

FIGURE 4A

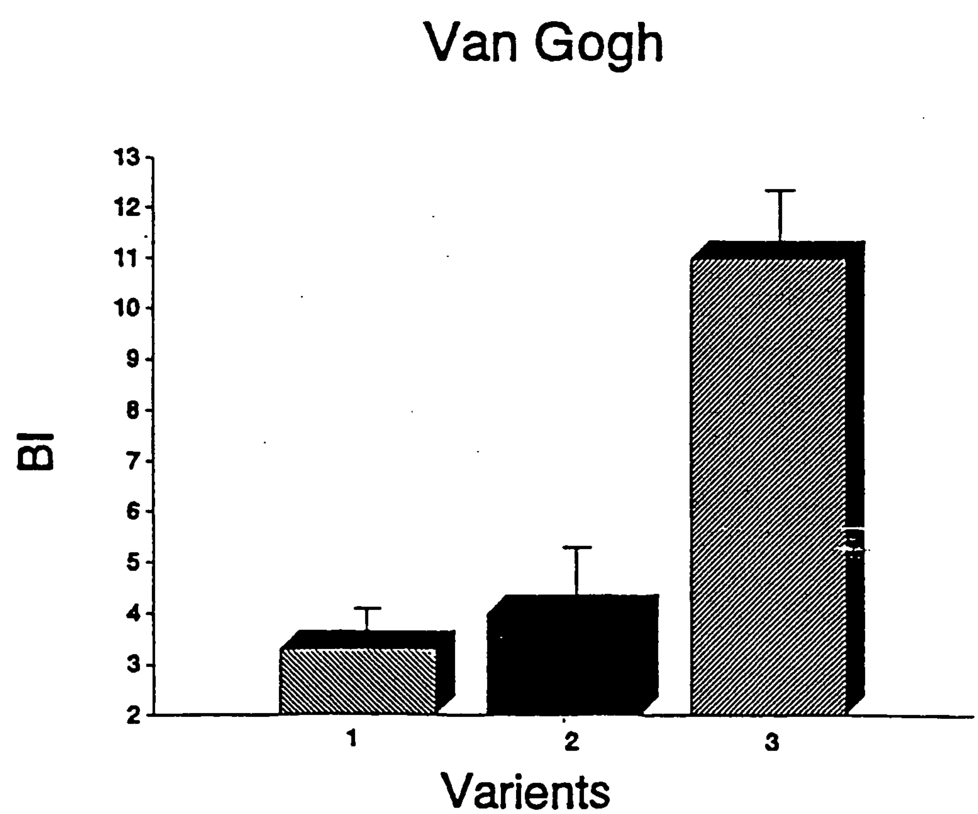

FIGURE 4B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 93/01988

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/53 C12N15/11 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOL. SUPPL. vol. 99, no. 1 , May 1992 page 88 HUNT, M.D, ET AL. 'A functional analysis of polyphenol oxidase (PPO) in Solanum-tuberosum using sense and antisense genes' see abstract 526 ---	1,5-7,9, 12-18,20
A	Embl sequence database rel. 32 Acc. no. M95196 13-6-92 ---	1,5-7,9, 12-18,20
A	Embl sequence database rel. 32 Acc. no. M95197 13-6-92 ---	1,5-7,9, 12-18,20
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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2

Date of the actual completion of the international search	Date of mailing of the international search report
8 December 1993	22 -12- 1993

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FOOD CHEMISTRY vol. 18 , 1985 pages 251 - 263 BATISTUTI, J.P., ET AL. 'Isolation and purification of polyphenol oxidase from a new variety of potato' see the whole document	1-20
A	GENE vol. 72 , 1988 pages 45 - 50 VAN DER KROL, A.R., ET AL. 'Antisense genes in plants: an overview' see page 48, right column	2-4
A	WO,A,92 11376 (AMYLOGENE) 9 July 1992 see page 9, line 32 - page 10, line 9	2-4
A	JOURNAL OF GENETICS AND BREEDING vol. 44 , 1990 pages 311 - 315 ROHDE, W., ET AL. 'Structural and functional analysis of two waxy gene promoters from potato' cited in the application see the whole document	2-4
A	THE PLANT CELL vol. 4, no. 2 , February 1992 pages 135 - 147 SHAHAR, T., ET AL. 'The tomato 66.3-kD polyphenoloxidase gene: molecular identification and developmental expression' cited in the application see the whole document	1-20
A	EP,A,0 240 208 (CALGENE) 7 October 1987 see the whole document	1-20
P,X	WO,A,93 02195 (CSIRO) 4 February 1993 see claims 25,26,28	1,12, 14-17,20
E	WO,A,93 15599 (CORNELL RESEARCH FOUNDATION) 19 August 1993 see example II	1,5, 7-17,20

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 93/01988

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		AU-A-	9114891	22-07-92
		EP-A-	0563189	06-10-93
EP-A-0240208	07-10-87	AU-A-	1301792	03-09-92
		AU-B-	618234	19-12-91
		AU-A-	7059787	01-10-87
		EP-A-	0458367	27-11-91
		JP-A-	62296880	24-12-87
		US-A-	5107065	21-04-92
		US-A-	4801540	31-01-89
WO-A-9302195	04-02-93	NONE		
WO-A-9315599	19-08-93	NONE		